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Viral Safety of a New Highly Purified Factor VIII (OCTATE)

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The inactivation of both transfusion-relevant and model viruses by modified pasteurisation (10 hours at 63°C in solution) has been evaluated following the established guidelines of the EU CPMP Ad Hoc Working Party on Biotechnology/ Pharmacy. This heat treatment was introduced into the manufacturing process of OCTAVI, a very high purity factor VIII concentrate stabilised only by von Willebrand factor, in the presence of a proprietary mixture of low molecular weight stabilisers. Both enveloped (human immunodeficiency virus, Sindbis virus, herpes simplex virus, pseudorabies virus) and nonenveloped viruses (poliovirus, Coxsackievirus, hepatitis A virus) were inactivated by this heating step by more than 4.7 $\log_{10}$. The combination of the solvent/detergent step used in the manufacture of OCTAVI with this modified pasteurisation leads to a double virus-inactivated factor VIII concentrate (OCTATE) with a viral safety distinctly superior to monoinactivated products.

KEY WORDS: validation studies, clotting factors, coagulation, HIV, double virus inactivation

INTRODUCTION

Whilst being of high therapeutic value, human plasma pools used for the manufacture of blood products can potentially be contaminated with a range of viruses. Those of major importance include the human immunodeficiency viruses (HIV-1 and HIV-2) and the hepatitis B (HBV), hepatitis C (HCV), and hepatitis D viruses [Gürtler, 1994]. Other viruses (e.g., parvovirus B19, hepatitis A virus [HAV], HTLV-I and HTLV-II, human herpes viruses types 6 and 7) are rarely associated with disease or only infrequently transmitted by pooled plasma products [Cuthbertson et al., 1991; Suomela, 1993]. It is of significance that the most common and serious blood-borne pathogenic viruses are lipid coated.

Approaches to improve the safety of plasma-derived products are based on three principles: a) careful selection of donors; b) careful screening of donated units for known infectious agents; and c) the use of validated manufacturing methods, which include specific steps designed to remove or inactivate viruses.

All donors are subjected currently to a wide battery of selection criteria and tests in order to determine their general suitability as donors. The criteria follow the guidelines of international, national, and local regulatory requirements. However, although donor selection can reduce significantly the virus load, it will not on its own guarantee noninfectious plasma pools. Therefore, all donated units are screened for viral markers. In contrast to HBV, where the plasma units are screened for antigen, HIV-1/2 and HCV infections are excluded by specific antibody screening. Hence, before seroconversion, these assays fail to detect infectious units ("diagnostic window"). Newly developed polymerase chain reaction (PCR) tests for these viruses appear to be highly sensitive, but are not yet suitable for routine screening of blood donations. Moreover, even the most sensitive assays currently on the market are unable to detect all donations capable of transmitting HBV ("low responder"). For HTLV-I/II, cytomegalovirus (CMV), and Epstein-Barr virus (EBV), there is no value in testing source plasma for antibody, as these agents are highly cell associated and are generally not transmitted by plasma products. Screening tests for other viruses (e.g., parvovirus B19) are not routinely available. Thus, to ensure the viral safety of plasma-derived products, specific additional steps resulting in either virus inactivation and/or virus removal have been introduced into manufacturing processes. These include solvent/ detergent treatment, heat treatment (in solution or of

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lyophilised products), partition methods (e.g., precipitation, chromatographic purification), and filtration methods, respectively.

The widespread introduction of solvent/detergent treatment into the manufacture of clotting factor concentrates [Horowitz et al., 1988; Burnouf-Radosevich and Burnouf, 1992], intravenous immunoglobulin preparations [Horowitz, 1989], and whole plasma [Piquet et al., 1992] has resulted in products with an excellent safety record [Cuthbertson et al., 1991; Goudemand et al., 1993; Horowitz, 1993; Hellstern et al., 1993]. However, only lipid-coated viruses are inactivated by this procedure. Since it cannot be excluded that blood-borne products treated by solvent/detergent alone may transmit nonenveloped viruses and in order to reduce the potential risk of infections due to them [Manucci, 1993], heat treatment for 10 hours at 63°C in solution in the presence of stabilisers was introduced into the manufacturing process of OCTAVI, a very high purity factor VIII concentrate stabilised only by von Willebrand factor [vWF; Schwinn et al., 1994]. The inactivation efficacy of this step was evaluated in a large number of virus validation studies with both transfusion-relevant viruses and several model viruses according to established EU guidelines [EEC Regulatory Document III/8115/89-EN, 1991; EEC Regulatory Document III/8379/89-EN, 1992].

MATERIALS AND METHODS

Viruses and Cells

HIV-1_{BRU} was obtained by transfection of the infectious DNA clone pBRU2 (source: MRC AIDS Reagent Project, National Institute for Biological Standards and Control, UK) into HUT-78 cells. The resulting virus was propagated on MT-4 cells. Pseudorabies virus (PRV; strain PRVdltk; ATCC VR-2074) was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and grown on Vero cells. Herpes simplex virus type 1 (HSV-1; strain MacIntyre) was obtained from ATCC (ATCC VR-539) and was propagated on Vero cells. Poliovirus type 1 (attenuated, strain Chat [WCH Wy-4B-S]) was obtained from ATCC (ATCC VR-192) and was propagated on Vero cells. Coxsackievirus type B6 (strain Schmitt) was obtained from ATCC (ATCC VR-155) and was propagated on Vero cells. SV40 (strain A2895) was obtained from ATCC (ATCC VR-305) and was propagated on CV-1 cells. Sindbis virus (strain New York) was obtained from the Institut Pasteur, (Paris, France) and was propagated on Vero cells. The HAV used was the cell culture-adapted human strain HM175/18f [Lemon et al., 1991]. To reduce the nonneutralisable fraction [Lemon and Binn, 1985], virus harvests were extracted with an equal volume of chloroform which was removed by adsorption to polystyrene. Stock solutions of all viruses were stored frozen at −80°C until use.

Vero cells (established kidney cell line from *Cercopithecus aethiops*) were obtained from ICN-Flow (Meckenheim, Germany). Cells were maintained in RPMI 1640 (ICN-Flow) supplemented with 5% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, and antibiotics. MT-4 cells (established T-lymphoma line) were a gift from Dr. Hagen von Briesen (Georg-Speyer-Haus) and grown in RPMI 1640 supplemented with 8.9% FCS, 2 mM L-glutamine, and antibiotics. BS-C-1 cells (established kidney cell line from *Cercopithecus aethiops*) used for HAV were maintained in minimal essential medium (MEM) supplemented with 2% FCS and antibiotics. CV-1 cells (established kidney cell line from *Cercopithecus aethiops*) were obtained from ATCC (ATCC CCL 70) and grown in RPMI 1640 supplemented with 8.9% FCS, 2 mM L-glutamine, and antibiotics.

Procedures

In-process samples of OCTATE factor VIII concentrate were obtained from Octapharma Produktionsgesellschaft mbH (Vienna, Austria). They were taken after the first virus inactivation step with 0.3% tri(n-butyl)phosphate/1% Tween 80 following the removal of the solvent/detergent mixture by anionic exchange chromatography. The factor VIII-containing fraction was subsequently stabilised by the addition of a proprietary mixture of amino acids and sugars. Representative volumes were heated to 63°C ± 0.5°C and then spiked with virus-containing cell culture supernatant in a ratio of 10:1. Aliquots were removed at different times and placed at 0°C. Except for the HAV studies, which were done at the University of North Carolina, all experiments were performed in the Georg-Speyer-Haus.

Virus Infectivity Assays

Serial tenfold dilutions of the virus stocks, the virus-spiked factor VIII preparations, and the heat-treated samples were titred on susceptible cells. Vero cells were used for HSV, PRV, poliovirus, Sindbis virus, and Coxsackievirus. MT-4 cells were used for HIV-1, CV-1 for SV40, and BS-C-1 for HAV. The adherent cell layers were grown in 96 well-plates, whereas MT-4 cells were maintained in 24 well-plates. In some experiments, undiluted samples were grown in culture flasks. Virus-induced cytopathogenicity was recorded visually at day 8 to 9 for HSV-1; at day 9 for Sindbis virus; at day 13 for PRV; at day 8 for poliovirus; at day 20 for SV40; and at day 12 for Coxsackievirus.

The replication of HIV-1 was monitored after 14–38 days by an antigen-capture assay (Organon-Teknika, Eppelheim, Germany), which was carried out according to the manufacturer's instructions.

HAV was detected by a radioimmunofocus assay [Lemon et al., 1983]. Briefly, Petri dish cultures of adherent BS-C-1 cells were inoculated with 0.25 ml of the sample dilutions. After incubating for 30 minutes, the cell sheets were washed to reduce cell toxicity and overlaid with agarose. The cells were screened 6 days later for HAV expression with radiolabelled anti-HAV anti-

TABLE I. Viruses Used for the Validation Studies

Virus	Genus	Genome	Lipid envelope	Resistance to physicochemical treatment
HIV-1	*Lentivirus*	RNA	Yes	Low
Sindbis virus	*Alphavirus*	RNA	Yes	Medium
HSV-1	*Simplexvirus*	DNA	Yes	Medium
PRV	*Varicellavirus*	DNA	Yes	Medium
Poliovirus-1	*Enterovirus*	RNA	No	High
Coxsackievirus-B6	*Enterovirus*	RNA	No	High
HAV	*Hepatovirus*	RNA	No	High
SV40	*Polyomavirus*	DNA	No	High

bodies by autoradiography; the titres were expressed as radioimmunofocus-forming units (rfu) per millilitre. Sample dilutions exhibiting cytotoxicity due to factor VIII and/or the stabilisers were excluded from the titre calculations. To increase the sensitivity of detection of infectious HAV, cell lysates from BS-C-1 cultures (ten replicates) inoculated with 1 ml of a 1:10 dilution of each sample were screened for HAV after 14 days by an antigen-capture assay.

Virus titres were determined by endpoint dilution and calculated according to Spearman-Kaerber [except in the case of HAV; Lemon et al., 1983]. The virus reduction factor was defined as the ratio of the virus load in the starting material prior to heat treatment to the virus load after virus inactivation. When virus replication was not observed, the detection limit was determined according to EU-guideline III/8115/89-EN [EEC Regulatory Document, 1991].

RESULTS

According to EU-guideline III/8115/89-EN, each modification of the current manufacturing process of plasma-derived products must be validated for its ability to remove or inactivate viruses. Validatable viruses fall into two categories: relevant and model viruses. Table I summarises the viruses used in this study.

Factor VIII:C eluates taken from the routine production of OCTATE were stabilised by low molecular weight additives to prevent denaturation and inactivation of both factor VIII and vWF during the heat treatment. They were heated to 63°C ± 0.5°C and then spiked with virus-containing cell culture supernatant. This procedure allowed an exact t_0 to be defined. Aliquots were taken at different times depending on the physical properties of the virus to be studied, and serial tenfold dilutions were titred on susceptible cells to determine residual infectivity. At low dilutions, evaluation of virus replication was limited due to cytotoxicity of the stabilisers (high osmolality). This effect could not be prevented in all cases even by a medium exchange after a short adsorption period (2 hours). Therefore, the inocula were not removed routinely except in the case of HIV-1 (more than 24 hours) and HAV (30 minutes). Virus titres are not changed significantly by this procedure (data not shown). To increase the sensitivity of detection of infectious viruses, cell cultures were maintained for long periods (see Materials and Methods). The results of the virus kill rates are summarized in Table II.

TABLE II. OCTATE: Virus Inactivation by Modified Pasteurisation

Virus	Target cells	Virus reduction factor (log_{10})
HIV-1	MT-4	≥9.9
Sindbis virus	Vero	≥8.4
HSV-1	Vero	≥7.7
PSV	Vero	≥5.3
HAV	BS-C-1	≥5.6
Poliovirus-1	Vero	≥9.8
Coxsackievirus-B6	Vero	≥4.7
SV40	CV-1	1.1

The results of these studies suggest that the viruses were stabilised partially by the additives used for factor VIII stabilisation. However, all viruses studied were inactivated completely within 600 minutes under these conditions, except SV40.

To assess the efficacy of this heat process, inactivation kinetics were studied for several of these viruses. Results for six viruses with different physical properties are summarised in Figure 1.

As expected, the lipid-enveloped viruses (HIV-1, Sindbis virus, HSV-1, PRV) were inactivated more rapidly than nonenveloped viruses. HIV-1 was inactivated completely within 120–140 minutes. Since some cultures were maintained for up to 38 days without any sign of virus replication, it is very unlikely that any infectious virus remained after this heat inactivation step.

The reduction in titre of the heat-sensitive HIV-1 and three other lipid-coated viruses, Sindbis virus (as model virus for HCV), PRV, and HSV-1 was a more or less linear process with similar kinetics in the presence of factor VIII, if the residual infectivity was presented in a logarithmic scale. However, complete inactivation of these viruses can be expected in practice. In contrast, the kinetics of inactivation of the nonlipid-coated viruses showed a significant "tailing" : the inactivation of virus infectivity followed a biphasic or multiphasic curve in which a rapid initial phase was followed by (a) slower phase(s). These results appear to reflect the

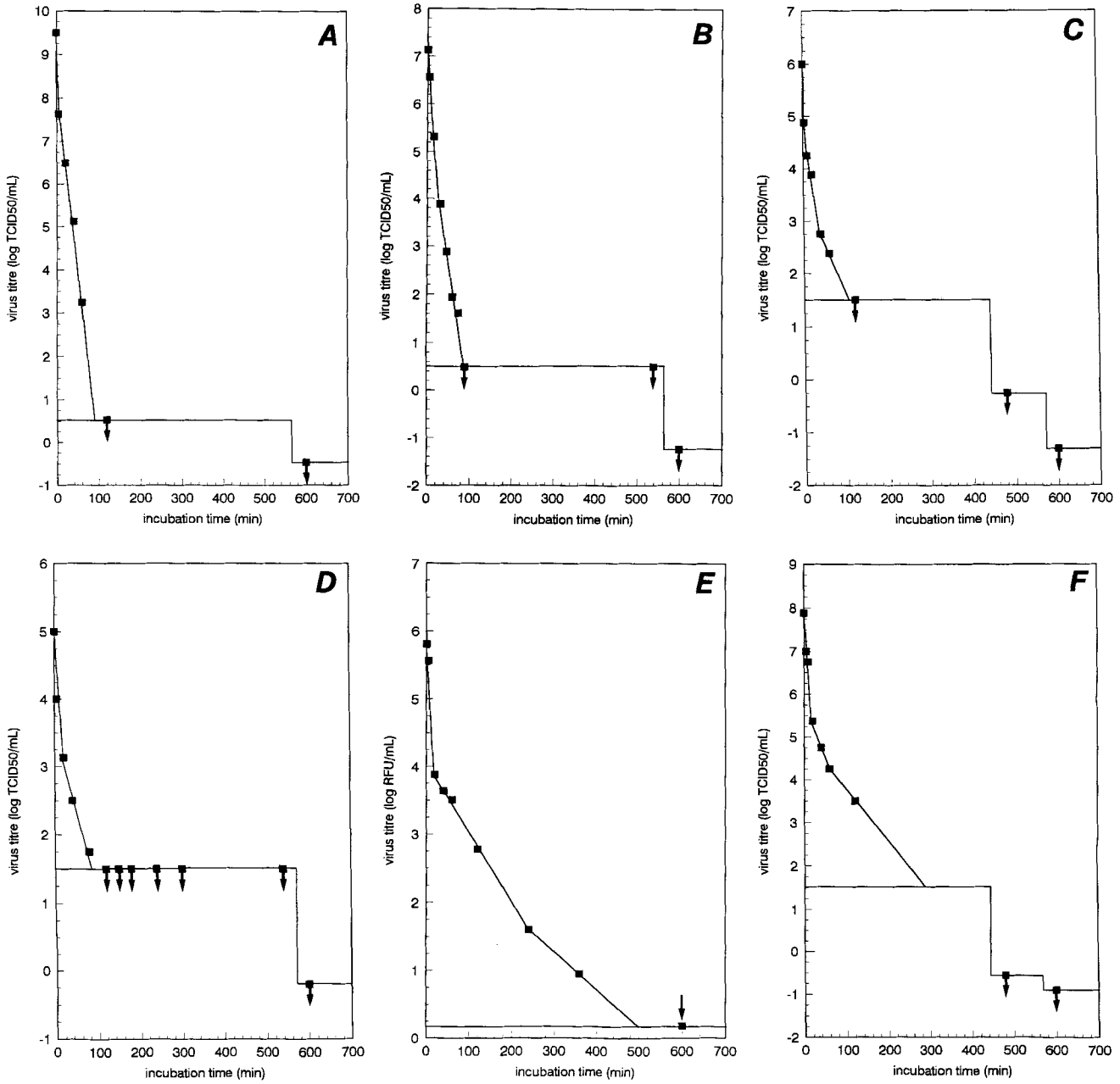

Fig. 1. Kinetics of virus inactivation at 63°C in stabilised factor VIII solution. **A:** HIV-1; **B:** Sindbis virus; **C:** HSV-1; **D:** PRV; **E:** HAV; **F:** poliovirus type 1. Arrows reflect that potential infectivity was beyond detection limit.

presence of two or more different subpopulations in the virus preparation.

Both HAV and poliovirus type 1 were inactivated rapidly by heat treatment when suspended in cell culture medium without stabilisers (data not shown). When suspended in the stabilised factor VIII, these picornaviruses were considerably more resistant to heat inactivation. HAV replication was not detectable by radioimmunofocus assay after 180 minutes at 63°C (residual titre <40 rfu/ml). However, infectious HAV was still identified in six of ten replicate cell cultures inoculated with 0.1 ml aliquots of a virus-spiked sample which had been heated at 63°C for 480 minutes (residual titre approximating 6 rfu/ml). After 600 minutes of heat treatment, no signs of virus replication were detectable even with this more sensitive assay. Thus, HAV was much more stable under these conditions than poliovirus type 1, which was completely inactivated within 4–5 hours below detection limit. A direct comparison of these poliovirus inactivation data with

those obtained in other laboratories is very difficult, because most likely different laboratory strains with different physical properties are used.

The model for nonenveloped DNA viruses, SV40, was minimally affected in its infectivity under the conditions employed (1.1 $\log_{10}$ reduction). This finding was observed in two pilot studies undertaken independently.

DISCUSSION

The viral safety of plasma-derived clotting factors is of major concern in the treatment of haemophiliacs. The factor VIII concentrate studied is manufactured by Octapharma using the solvent/detergent method for virus inactivation. Solvent/detergent treatment has an excellent safety record concerning the major transfusion-relevant viruses (HIV, HBV, HCV) and is used worldwide. Due to its specific mode of action, this method is not expected to be effective against nonenveloped viruses [Lemon et al., 1994]. Recently, several reports in the literature indicated that the nonenveloped HAV may have been transmitted by transfusion of factor VIII concentrates which had undergone virus inactivation by solvent/detergent alone [Manucci, 1992; Lawlor et al., 19984; Peerlinck et al., 1994]. Therefore, a heat step for 10 hours at 63°C in solution was introduced into the manufacturing process of OCTAVI. This approach departed from conventional pasteurisation because the temperature necessary to inactivate the heat-resistant picornaviruses was reported to be greater than 60°C [Siegl et al., 1984]. The results presented here demonstrate that HAV was inactivated completely under these modified conditions by $\geq$5.63 $\log_{10}$. It was found that the time required for complete virus inactivation was substantially increased, when HAV was heated at 60°C for 10 hours in the presence of low molecular weight stabilisers, which corresponds to conventional pasteurisation [Murphy et al., 1993].

The ability to reduce potential viral contaminants must be demonstrated for the entire manufacturing process by the validation of individual steps in downstream processing for their individual efficacy of virus removal and/or virus inactivation. The requirements for the performance of virus validation studies are documented in two EU-guidelines (EEC Regulatory Document, 1991, 1992). In practice it is impossible to validate the inactivation of virus under the conditions of a manufacturing process. The use of infectious virus as a spike would contaminate the manufacturing equipment to an irresponsible degree and thus contradict Good Manufacturing Practice (GMP). In addition, it is very difficult in practice to make available the amount of virus required on a scale corresponding to large batch sizes. Therefore, the validation experiments must be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in cooperation with production bioengineers who are involved in designing and preparing a representative scaled-down version of the inactivation process. Three aspects have to be taken into consideration: 1) a high titre is desirable to study the capability of the virus inactivation step; 2) the appropriate intermediate product taken from the routine production just prior to the process to be analysed should be used as starting material; 3) changes in the chemical composition of the starting material resulting from the addition of the virus-containing cell culture supernatant should be minimised. In practice, not more than 10% (v/v) of virus-containing cell culture supernatants should be used to spike the factor VIII in-process sample.

A major issue in carrying out validation studies is the selection of the viruses to be used. Such viruses fall into two catagories: relevant and model viruses. Relevant viruses are known to, or likely to, contaminate the source material or other materials used in the production process. As human clotting factors have been contaminated by HIV, this virus is mandatory according to the EU-guideline III/8115/89-EN as a relevant virus for products derived from human blood. However, it is impossible to infect cultured cells with some relevant viruses such as HBV. Similarly, no good cell culture system exists for propagation and titration of HCV. If the use of relevant viruses is practically restricted, then validation studies should be performed with so-called model viruses which encompass viruses with a wide range of physicochemical properties. These include related DNA and RNA viruses, both enveloped and nonenveloped. Viruses which can be grown to high titre are desirable, although this may not always be possible. In addition, there should be an efficient and reliable assay for the detection of the viruses used. It is important that the strains used should retain the properties of the wild-type virus. In the case of HAV, there are a total of only three amino acid changes in the capsid proteins of HM175/18f compared with the wild-type HM175 virus [Lemon et al., 1991]. Although the HM175/18f strain has excellent growth properties in cell culture, it retains the antigenicity and thermal stability characteristics of low-passage HAV.

Reduction in virus infectivity may be achieved by removal of virus particles or by inactivation of the infectious agent. In both cases, validation is carried out following a similar approach. Whenever possible, it is desirable to obtain kinetic analysis of viral inactivation, in order to determine the slope of the curve and to extrapolate the time theoretically required to inactivate the total virus population.

The reduction of infectious HIV-1 and the three lipid-coated model viruses (Sindbis virus, PRV, and HSV-1) in the presence of factor VIII was a more or less linear process with similar kinetics, if the residual infectivity was shown in a logarithmic scale. These results suggest a thermal stability comparable to HIV-1 under these conditions. Given to time points taken for the kinetics of these viruses, a finer distinction between their relative stabilities could not be made. However, complete inactivation of these viruses can be expected in practice, since the heat treatment continues for 10 hours without taking into account the time required to heat up and cool down the stabilised factor VIII preparation.

In contrast, the kinetics of the nonlipid-coated viruses followed a least biphasic curve in which a rapid initial phase was followed by a slower phase. These results reflect the presence of two or more different subpopulations in the virus preparations. The more heat-resistant fraction(s) might result from virus aggregates or perhaps differences in capsid structure due to selection of a mutant subset [Lemon and Binn, 1985]. HAV was much more stable under these conditions than poliovirus type 1, which was completely inactivated below detection limit within 4 to 5 hours. A direct comparison of these poliovirus inactivation data with those obtained in other laboratories is very difficult, because most likely different laboratory strains are used.

In contrast to the nonenveloped RNA viruses (poliovirus, Coxsackievirus, and HAV), the model for nonenveloped DNA viruses, SV40, was affected minimally in its infectivity under the conditions employed (1.1 $\log_{10}$ reduction). This finding was observed in two independently performed pilot studies. Possibly as a consequence of its structural simplicity, the virion is known to be extremely resistant to inactivation in the presence of stabilisers.

The overall virus reduction factor for a manufacturing process is determined from the sum of the individual virus reduction factors based on individual process steps of different physicochemical methods (EEC Regulatory Document, 1991). The two completely different physical inactivation principles, solvent/detergent inactivation and heat treatment, employed in the manufacture of OCTATE are likely to be additive. To guarantee viral safety of plasma-derived products, the overall reduction factor should be substantially greater than the maximum possible virus load which could occur potentially in the source material. Viraemia in patients with primary HIV-1 infection reaches titres of at least 3 to 4 $\log_{10}$ $TCID_{50}$/ml [Daar et al., 1991]. Viraemia in monkeys infected experimentally with a cell culture-adapted HAV reached titres in excess of 5 $\log_{10}$ rfu/ml serum [Tabor et al., 1983]. Set against these figures, the manufacturing process of OCTATE is capable of eliminating at least 15 $\log_{10}$ for HIV-1 [Statens Serum Institut, personal communication]. At least 11 $\log_{10}$ infectious HAV could be cleared by mechanisms of virus removal, virus inactivation by the heat treatment evaluated here, and immune neutralisation [Lemon et al., 1994].

In conclusion, the combination of solvent/detergent treatment with a modified pasteurisation step leads to a highly purified factor VIII concentrate (OCTATE) with a viral safety which is likely to be superior to monoinactivated products.

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